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Original Article

AIEOP-BFM Consensus Guidelines 2016 for Flow Cytometric Immunophenotyping of Pediatric Acute Lymphoblastic Leukemia

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Immunophenotyping by flow cytometry (FCM) is a worldwide mainstay in leukemia diagnostics. For concordant multicentric application, however, a gap exists between available classification systems, technologic standardization, and clinical needs. The AIEOP-BFM consortium induced an extensive standardization and validation effort between its nine national reference laboratories collaborating in immunophenotyping of pediatric acute lymphoblastic leukemia (ALL). We elaborated common guidelines which take advantage of the possibilities of multi-color FCM: marker panel requirements, immunological blast gating, in-sample controls, tri-partite antigen expression rating (negative vs. weak or strong positive) with capturing of blast cell heterogeneities and subclone formation, refined ALL subclassification, and a dominant lineage assignment algorithm able to distinguish "simple" from bilineal/"complex" mixed phenotype acute leukemia (MPAL) cases, which is essential for choice of treatment. These guidelines

Additional Supporting Information may be found in the online version of this article.

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are a first step toward necessary inter-laboratory standardization of pediatric leukemia immunophenotyping for a concordant multicentric application. **© 2017 International Clinical Cytometry Society**

Key terms: standardization; flow cytometry; leukemia; pediatric; immunophenotyping:

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INTRODUCTION

Immunophenotyping by flow cytometry (FCM) is a cornerstone of the diagnostic work-up of leukemia. Recent years have rendered important improvements and fine-tuning of the diagnostic pathways used to assess leukemia, the technical armamentarium of FCM, and the sub-classification based on detailed biological insights—as summarized in the 2008 and 2016 revisions of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia (1–4). However, general implementation of these advancements into daily practice in a comprehensive, standardized and quality controlled way is lagging behind (5–7).

In the run-up to the multinational treatment trial for pediatric acute lymphoblastic leukemia (ALL), jointly designed by the Associazone Italiana Ematologia Oncologia Pediatrica (AIEOP) and the Berlin-Frankfurt-Münster (BFM) group, AIEOP-BFM ALL 2009, the nine involved national FCM reference labs—providing service to an annual recruitment of approximately 1000 children with ALL—were induced by the trial authorities to elaborate a common immunophenotyping standard-operating-procedure (SOP) as an up-date to previously used standards (8–10). Several issues of technology and classification were put forward under the premises that it is reasonable to conserve local practices—based on ample experience—but with some adaptations to meet corporate requirements. First, a methodological transition was specified to respect the advent and chances of multicolor FCM toward (i) improved immunological blast cell gating with the ability to explore smaller and/or more heterogeneous populations than previously possible, (ii) advanced resolution and sensitivity in determining cellular antigen expressions, (iii) inclusion of novel markers or marker combinations with relevance for subclassification and/or outcome prognostication, and (iv) introduction of a detailed, semiquantitative capturing of expression types (1,2,11–15). Accordingly, it was decided to introduce the rating of weak and strong expression—deliberately applied by the WHO 2008 guidelines—into AIEOP-BFM practice (16). Toward this end, we needed to clarify uncertainties regarding clear-cut thresholds for a concordant application throughout many labs. Second—and related to this previous aspect—we needed to specify hematopoietic lineage assignment which remains the diagnostic mainstay for treatment selection and trial inclusion. In particular, the topic of mixed phenotype acute leukemia (MPAL; according to WHO 2008/2016), previously captured somewhat differently as biphenotypic acute leukemia (BAL) by the European Group for the Immunological Characterization of Leukemias (EGIL), has perturbed clinicians significantly with respect to best therapeutic options (9,16–24).

The standardization project debuted in July 2009 and comprised several task group meetings (literature search and topic discussions, joint case reviews, preliminary concordance assessments of local case cohorts, guideline drafting) and culminated in the internal release of a first consensus guideline in December 2012, which is delineated herein in its most recent 2016 version.

CONSENSUS GUIDELINES

Antibody Panel

The consensus panel (Table 1) is designed to fulfil EGIL and WHO 2008/2016 requirements for ALL-subtyping, MPAL (16), BAL (9,10), as well as early T-cell precursor (ETP) leukemia discrimination (12,25,26).

A multicolor staining approach is compulsory (i.e., $\geq$4 colors; 6-color approach recommended) and should be based on a CD45-backbone plus markers against (both) lymphoid lineages in each tube to pull out leukemic and potential control cells. The choice of fluorochrome-conjugated antibody clones, vendors, and tube combinations is largely left to individual lab's expertise with the exception of a strong recommendation regarding prominent lineage-defining markers like the intracellular myeloperoxidase antibody (iMPO, clone 8E6), the antibodies against iCD22 and iLysozyme (clones RFB4 and LZ-2, respectively), as well as iCD3 (clones UCHT1 or SK7). The necessity of panel standardization regarding certain antigens has been investigated in a still ongoing validation process in which we have been comparing the individual antibody panels of several centers against an antibody panel completely standardized between laboratories (M.N. Dworzak et al. manuscript in preparation). In case of applying a sequential staining approach (using a screening tube as well as a narrowed, lineage-specific secondary panel), the screening must include the markers CD19, CD10, iCD79a, iCD22, iCD3/CD3, CD7, iMPO, and CD45 to

warrant dominant lymphoid-lineage determination (see below) in all cases.

Optional/recommended markers add to drawing a comprehensive picture of the individual leukemic phenotypes and fulfil two alternative purposes: First, antigens like CD11a, CD38, CD58, CD99, and CD123 have a strong rationale toward leukemia follow-up and minimal residual disease (MRD) assessment (27–31), which is a decisive task for immunophenotyping in AIEOP-BFM trials. Second, they are associated with genetic lesions or novel clinical subtypes potentially impacting on outcome. Genotype-associated markers are for example CD123 (hyperdiploidy), CD66c (hyperdiploidy, *BCR/ABL*), NG2 (*MLL*-rearrangements), CRLF2 (*CRLF2*-rearrangements), or lack of CD44 positivity (*TEL/AML1*; also in mature B-ALL with *MYC*-translocation) (1,15,31–39). The consortium, however, considers the fact that per AIEOP-BFM trial practice immunophenotyping is generally not used as gateway for genetic assessments. More important than genotype surrogates, the consensus antibody panel allows capturing novel clinical ALL subtypes for which specific genetic lesions have not been defined. This includes ETP and the newly described B-cell precursor (BCP)-ALL entity with a propensity to an early switch to the monocytic lineage (40). Such switch-ALL, apart from being CD2-positive in many cases, is most efficiently determined at diagnosis based on CD371 positivity (M.N. Dworzak et al. manuscript in preparation).

Table 1
The AIEOP-BFM consensus antibody panel for pediatric ALL

Mandatory and optional markers (each combined with CD45)	
Intracellular[a,b]	iCD3, iCD22, iCD79a, iIgM (μ-chain), iLysozyme, iMPO
Surface[a]	CD2[c], CD3, CD5, CD7; CD10, CD19, CD20; CD11c, CD11b, CD13, CD14, CD15, CD33, CD64, CD65[d], CD117; CD34, (CD45), CD56, HLA-DR if T-ALL: CD1a, CD4, CD8, TCRαβ, TCRγδ if B-IV suspected: κ-chain, λ-chain (surface staining after pre-washing or intracellular)
Optional / Recommended	all cases: NG2[e], CD371[c,f] if BCP-ALL: CD11a[c], CD22, CD24, CD38, CD44, CD58, CD66c, CD123[c], CRLF2[c,g] if T-ALL: CD99, iTdT if BAL according to general panel: CD24, iTdT

[a]Mandatory markers for WHO, EGIL, ETP classifications.
[b]Prefix "i" stands for intracellular staining.
[c]Phycoerythrin-conjugate (PE) recommended.
[d]Available only labelled with fluorescein isothiocyanate (FITC).
[e]Clone 7.1.
[f]Clone 50C1.
[g]Clone 1D3.

Sample Priority, Preparation, Staining and Acquisition

Bone marrow (BM) is preferred over peripheral blood for assessing the immunophenotype at diagnosis. The latter may be used in case BM is not available or of poor quantity/quality or in case of massive peripheral blastosis. In principle, a stain-lyse procedure is used (27). Additional staining with a live-cell-permeant nucleated-cell dye is recommended to allow distinguishing non-nucleated events (erythrocytes, debris) from nucleated cells (NC) (27). This is particularly relevant to compensate for diminished sample quality after prolonged transportation to central reference laboratories (by excluding residual non-nucleated events from blast cell assessment and enumeration among NC) and specifically in ALL cases with a very low CD45 expression on blasts (which fall into the CD45 area where non-nucleated events are found). For cellular permeabilization and intracellular staining, we use commercial reagents according to the manufacturers' recommendations (27,41,42).

Sample acquisition on flow-cytometers should measure a minimum of 30,000 NC per tube. This warrants that—at a stipulated sensitivity of 1%—also minor blast subpopulations as well as residual normal lymphocytes (to be used as internal control, see below) can be immunophenotyped with sufficient precision and narrow CV by acquiring several hundreds of such events (43,44). Necessary procedures for quality control of the instrumental setup and over-time performance by recording setup and tracking beads according to manufacturer's recommendations are described elsewhere (27,44,45).

Analysis and Gating Strategy

Immunological blast gating is applied using a CD45/SSC backbone strategy for delineating the blast cell region in the CD45-low/SSC-low area (5,46), plus lineage-defined gating as appropriate. Doublets exclusion of events with increased FSC-Width is mandatory because such events frequently exhibit false expressions (Supporting Information Fig. 1; Ref. 44). Assessment of individual antigen expression is done preferably using histograms, contour plots, or pseudocolor density plots, rather than with ordinary dot plots or parameter band diagrams. Usage of plots with biexponential or logical display is specifically advantageous for investigating low intensities and negative values (44,47). A negative region is then defined for each antigen by in-sample cell controls (i.e., mature, cross-lineage lymphocytes gated in plots with CD45 and/or cross-lineage markers) which do not express the antigen. This approach is currently seen as one of the most appropriate control measures because of the exposure of all populations to identical conditions (44,48,49). Secondly, we address autofluorescence. To this end, we use unstained control tubes (one permeabilized for comparison with intracellular stainings and one non-permeabilized for surface antigens) (48,49). However, to respect that different cell types within a sample may exhibit different background fluorescence (Supporting Information Fig. 2; Refs. 48 and 49) we also recommend the use of further two control tubes (one

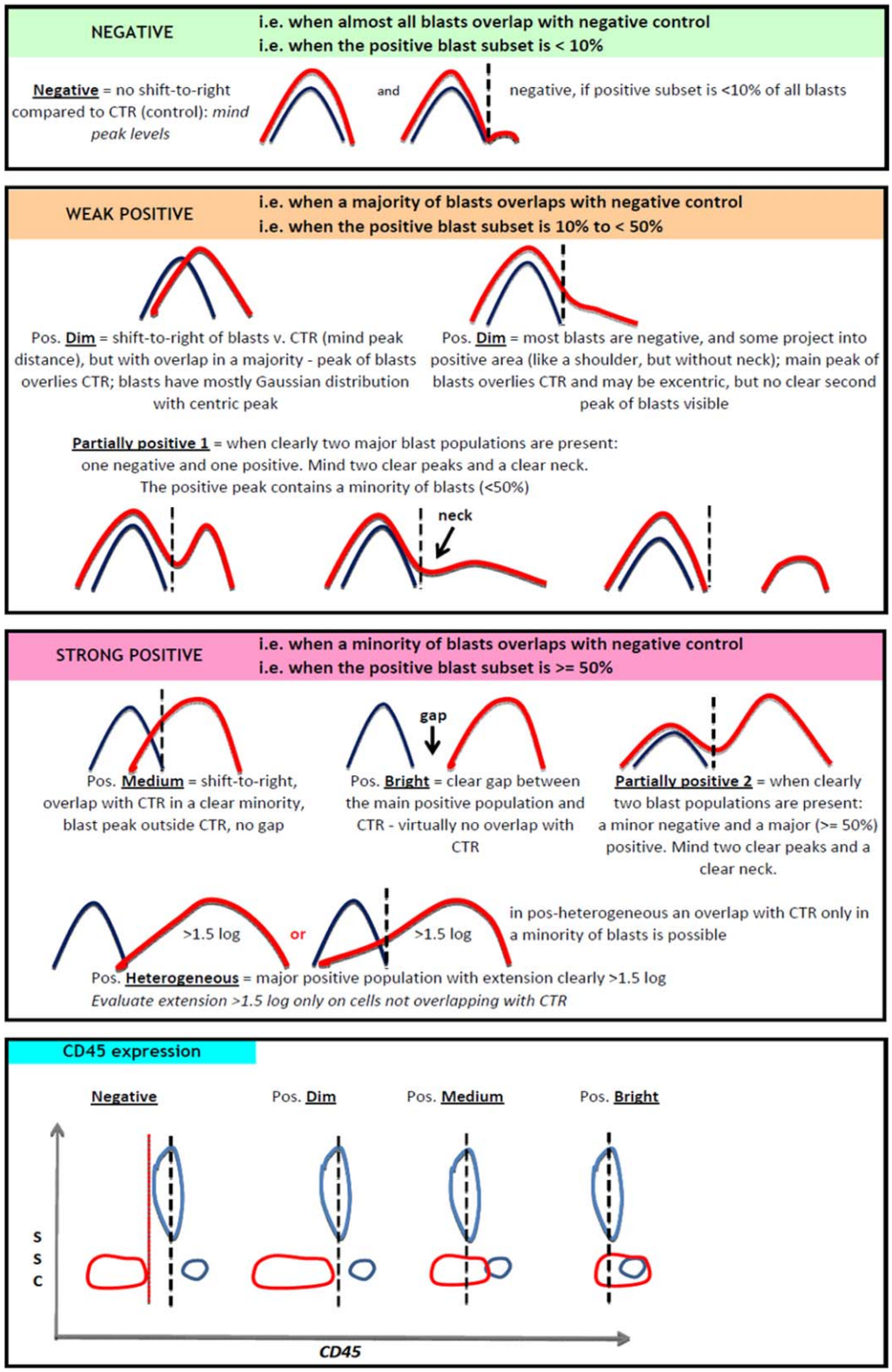
NEGATIVE
i.e. when almost all blasts overlap with negative control
i.e. when the positive blast subset is < 10%
Negative = no shift-to-right compared to CTR (control): mind peak levels
and
negative, if positive subset is <10% of all blasts
WEAK POSITIVE
i.e. when a majority of blasts overlaps with negative control
i.e. when the positive blast subset is 10% to < 50%
Pos. Dim = shift-to-right of blasts v. CTR (mind peak distance), but with overlap in a majority - peak of blasts overlies CTR; blasts have mostly Gaussian distribution with centric peak
Pos. Dim = most blasts are negative, and some project into positive area (like a shoulder, but without neck); main peak of blasts overlies CTR and may be excentric, but no clear second peak of blasts visible
Partially positive 1 = when clearly two major blast populations are present: one negative and one positive. Mind two clear peaks and a clear neck. The positive peak contains a minority of blasts (<50%)
neck
STRONG POSITIVE
i.e. when a minority of blasts overlaps with negative control
i.e. when the positive blast subset is >= 50%
Pos. Medium = shift-to-right, overlap with CTR in a clear minority, blast peak outside CTR, no gap
gap
Pos. Bright = clear gap between the main positive population and CTR - virtually no overlap with CTR
Partially positive 2 = when clearly two blast populations are present: a minor negative and a major (>= 50%) positive. Mind two clear peaks and a clear neck.
>1.5 log
or
>1.5 log
in pos-heterogeneous an overlap with CTR only in a minority of blasts is possible
Pos. Heterogeneous = major positive population with extension clearly >1.5 log
Evaluate extension >1.5 log only on cells not overlapping with CTR
CD45 expression
Negative
Pos. Dim
Pos. Medium
Pos. Bright
SSC
CD45

FIG. 1. **Overview scheme and definitions of antigen expression rating according to the AIEOP-BFM consensus guidelines.** Note that, for the tripartite major expression capturing (negative, weak, strong positive) as well as for the *Bethesda-style* fine-rating, cross-lineage lymphocytes are used as negative-control populations, with the exception of a few broadly expressed antigens like CD45, which receive adapted fine-rating. For CD45 we recommend assessment of its expression on the blast subset (red) versus residual granulocytes (blue; high SSC) and mature lymphocytes (blue; low SSC).

Table 2
The AIEOP-BFM dominant lineage assignment[a]

Lineage	Criteria	Antigens
BCP-ALL	≥2 positive of:	CD19[b]; CD10, (i)CD22, iCD79a
T-ALL	all 3 of:	(i)CD3[pos c], CD7[pos]; iMPO [negative or weak]
AML	≥2 positive of:	iMPO, CD13, CD33, CD64, CD65, CD117
	and:	BCP-/T-ALL criteria not met

[a]Of note, these markers are relevant for dominant lineage assignment, but are insufficient for a thorough description of leukemic immunophenotypes.

[b]BCP-ALL needs strong positivity in ≥2 of the four antigens – in the rare case of CD19-negativity, specifically CD10 must be strong positive. Mind that rare cases of MLL-rearranged BCP-ALL may drop out of this scheme due to biology-inherent lack of CD10, as well as weak (i)CD22 and iCD79a expression (CD19 is then usually strong positive).

[c]For T-ALL, iCD3 positivity must be either strong, or if rated weak, CD2 and/or CD5 should be any positive in addition. Surface CD3 expression needs to be tested in addition.

permeabilized and one non-permeabilized) stained only with the CD45 reagent. With these tubes we discriminate blasts from other cell populations according to differential CD45 expression and then estimate the potentially different autofluorescence of blasts and control cells in the unstained channels. The higher level of the two background regions (from in-sample cell control or autofluorescence) should be regarded as more relevant. With paucicellular specimens, where we need to prioritize tubes in order to run the full antigen panel, we prefer the control tubes containing only the CD45 reagent because giving most information except for CD45 background itself. It should be emphasized that we do not recommend the usage of isotype controls as has been established before by several authoritative publications (43,44,47–49). This omission relies on the use of well-described internal control populations available now that whole blood or BM samples are used for FCM. However, in case of totally missing cross-lineage control cells as well as in particular in situations of suspected artifactual results, fluorescence minus one (FMO) tests are pertinent. These are also advised for learning expression patterns with new combinations of antibodies (44).

Definition of Antigen Expression

The consortium follows in its guidelines recent developments in the field with respect to (i) increased technical sensitivity which renders previously applied higher cut-offs for positivity (e.g., 20%) obsolete (44), and (ii) the rejection of reporting antigen expression solely by percentages (1,2,5,44,50). Antigen expression of the gated blasts is determined by assessing the fluorescence shift and distribution pattern of the leukemic population against appropriate control as delineated before (1,2,5,50). The amount of deviation from negative is further used to differentiate positivity into two major categories (weak and strong, see Fig. 1). Only in borderline situations the prevalence of non-overlap of blasts with the negative control population is quantified (instead of using, for example, the standard deviation of median fluorescence intensity; Refs. 1,50). A threshold marker is then placed to delimitate the negative region as defined above and to assess the proportion of blasts outside this region (Fig. 1; Supporting Information Fig. 3). A threshold ≥10% of gated blasts is used for all antigens (independently from surface or intracellular location) to consider an antigen as "positive." Hence, positivity is only accepted once a fluorescence shift is large enough to exceed potential background variability (1,50–52). However, threshold markers are not used for partitioning of otherwise single blast populations for reporting of proportions of cells aside from the marker.

In full concordance with the *WHO-style* tri-partite consensus rating (negative-weak-strong), a more detailed further description of antigen distribution and intensity is recommended (e.g., *Bethesda-style* rating; Fig. 1). According to the latter, in particular immunophenotypic subclones of leukemic blasts can be delineated upon description of only partially expressed markers. This may occur in both, the weak and the strong positive expression categories. Such fine-rating makes use of a descriptive and semi-quantitative scale rather than an exact enumeration procedure (2). The focus of such a more detailed interpretation is more on robustness in daily routine and less on biologic accuracy. Accordingly, an exception exists with respect to broadly expressed pan-leukocyte markers such as CD45 and CD11a, as well as CD38, CD58, and CD99. With those, the system of in-sample negative-control lymphocyte subsets cannot easily be applied because lymphocytes of all lineages are positive with these markers. In case of CD45 and CD11a, expression on blasts is compared to the unstained channel control (negative) as well as to residual mature neutrophils (SSC^{high} and/or $CD15^{bright}$) in the sample which are positive (Fig. 1). In case of CD38, CD58, and CD99, blasts should be compared to those residual SSC^{low} lymphocyte subsets with a higher expression of the marker: if lower than those but not negative, rating is *dim* positive; if similar in expression, the rating is *medium*; if stronger, the rating is *bright* (e.g., this is the type of CD38 expression in plasma cells); if broadly overlapping, *heterogeneous*. Mind that the latter rating is related to a certain (broad) pattern of expression of a single marker. This has to be distinguished from the term *blast population heterogeneity* (see below), which is used to describe immunophenotypic subset formation in a given case of leukemia.

Blast Percentage Recording

The percentage of blasts among total NC (see above; Ref. 27) of the sample is recorded. Notably, formal diagnosis of leukemia and treatment indication usually depends on morphologic blast enumeration respecting conventional thresholds (e.g., ≥25% as per AIEOP-BFM ALL 2009 protocol, or 20% as per WHO 2008

Table 3
The AIEOP-BFM subclassification of ALL[a]

Subtype	Discriminators	Remarks
B-I (pro-B)	$CD10^{neg}$	BCP-ALL lineage criteria fulfilled
B-II (common)	$CD10^{pos}$	/
B-III (pre-B)	$iIgM^{pos}$	$CD10^{neg\ or\ weak\ pos}$ may occur[b]
B-IV (mature B)	κ- or λ-$chain^{pos}$	may occur with FAB L1/L2 morphology[c]
T-I (pro-T)[d]	only $iCD3^{pos}$ and $CD7^{pos}$	T-ALL lineage criteria fulfilled
T-II (pre-T)	$\geq$1 of $CD2^{pos}$, $CD5^{pos}$, $CD8^{pos}$	surface (s) $CD3^{weak\ pos}$ allowed[e]
T-III (cortical T)	$CD1a^{pos}$	$sCD3^{weak}$ may occur[e]
T-IV (mature T)	$CD1a^{neg}$ and $sCD3^{pos}$	$sCD3^{strong}$, or $sCD3^{weak\ pos}$ with TCR^{pos}
ETP (only additive to T-I or T-II)	$CD1a^{neg}$, $CD8^{neg}$ usually $CD5^{neg\ or\ weak\ pos}$ and $\geq 1^{pos}$ of HLADR, CD11b,13,33,34,65,117	if $CD5^{strong\ pos}$: $\geq 2^{pos}$ of HLADR, CD11b,13,33,34,65,117; $sCD3^{weak\ pos}$ may occur[e]

[a]Adapted from Refs. 8 and 9.
[b]$CD10^{neg/weak}$ B-III is frequently associated with MLL-rearrangements (12).
[c]Light-$chain^{pos}$ cases without FAB L3-morphology and without *MYC*-translocation are eligible for conventional ALL treatment, and thus must be separated from Burkitt-type mature B-ALL (40–43).
[d]T-I is very rare and can be reported together with T-II (as T-I/II).
[e]Dim or even more frequently partial surface positivity with CD3 (e.g., in a minor blast subpopulation) occurs when sensitive methodology is used and should not mislead to diagnose mature T-ALL in the absence of TCR expression.

definition). This threshold requirement is, however, independent of the process of immunophenotyping of the leukemia. Hence, the leukemic immunophenotype can be determined as long as the blast population can be clearly distinguished from normal background cells regardless of numerical blast-percentage cut-off (53). We consider the fact of nearly systematic hemodilution of BM aspirations collected for immunophenotyping, which indeed hampers fulfilling criteria derived from microscopic traditions. In such case, with a malignant blast percentage <25% by FCM, however, the immunophenotypic report should not anticipate formal diagnosis of ALL but state, for example, "malignant lymphoblasts suggestive of ALL with a low blast percentage."

Reporting of Blast Immunophenotype – Blast Population Heterogeneity

The leukemic cell population can exhibit heterogeneities. Therefore, it must strictly be avoided that immunophenotypic reporting lumps together features of immature blasts and maturing cells (not an issue in ALL, but typical for myeloid leukemias with maturation), as well as of heterogeneous blast populations by reporting many antigens as partially positive. Rather, samples must vigilantly be assessed for signs of more than one immunophenotypic blast "clone." Such cases should be classified by an extra label as "heterogeneous blast population." Two major situations correspond to this: First, leukemia with separate, immunophenotypically largely non-interconnected blast clones (typically occurring as bilineal). In such case, the detailed phenotype should be reported for each blast clone separately (including percentage among NC, lineage and subtype assignment, antigen expression pattern, for example, each in the *Flow Diagnostics Essential Code* [FDE-code] format; Ref. 53). Second, leukemia with a branched, interrelated blast population, in which such heterogeneity is captured by reporting antigens as partially positive. To qualify for the label "heterogeneous blast population," such cases must either fulfil the MPAL/BAL- or ETP-criteria by inter-lineage clonal heterogeneity (see Supporting Information Fig. 3), or exhibit intra-lineage clonal heterogeneity that would lead to different ALL subtype classifications (see Supporting Information Fig. 4A and B) when assessing blast subsets separately. To this end, the following antigens are of interest in ALL when only partially positive: CD10, (i)IgM, κ-chain, λ-chain, CD1a, CD3, CD5, TCRαβ, TCRγδ.

In addition, very rare situations of interlineage heterogeneity exist where both, clearly separate blast subpopulations of different lineages as well as further subsets interconnecting the former occur (see Supporting Information Fig. 4C), which can best be summarized as (bilineal) MPAL cases with "complex immunophenotype."

Possible are also situations where an antigen is rated clearly negative in the great majority of blasts, but a minute (<10%) subset is unambiguously positive. This is typical for CRLF2, NG2, and other antigens (see Supporting Information Fig. 5). Such an antigen expression on very minor subsets of blasts should not be rated partially positive according to our definition, but deserves reporting because of potential biologic relevance (e.g., by "blast heterogeneity" and by a description of that subpopulation in the diagnostic summary).

Dominant Lineage Assignment

Inclusion into AIEOP-BFM ALL treatment trials is based on morphologically and genetically defined lymphoblastic leukemia with a dominant ALL-immunophenotype according to the AIEOP-BFM lineage assignment criteria (Table 2) adapted from Mejstrikova et al. (20). This is irrespective of whether or not a case fulfils also criteria of MPAL (WHO 2008/2016, Refs. 3,4, and 16; Supporting Information Table 1) or BAL (EGIL, Refs. 9 and 10; Supporting Information Table 2)—even in case of MPO positivity—in a single blast population.

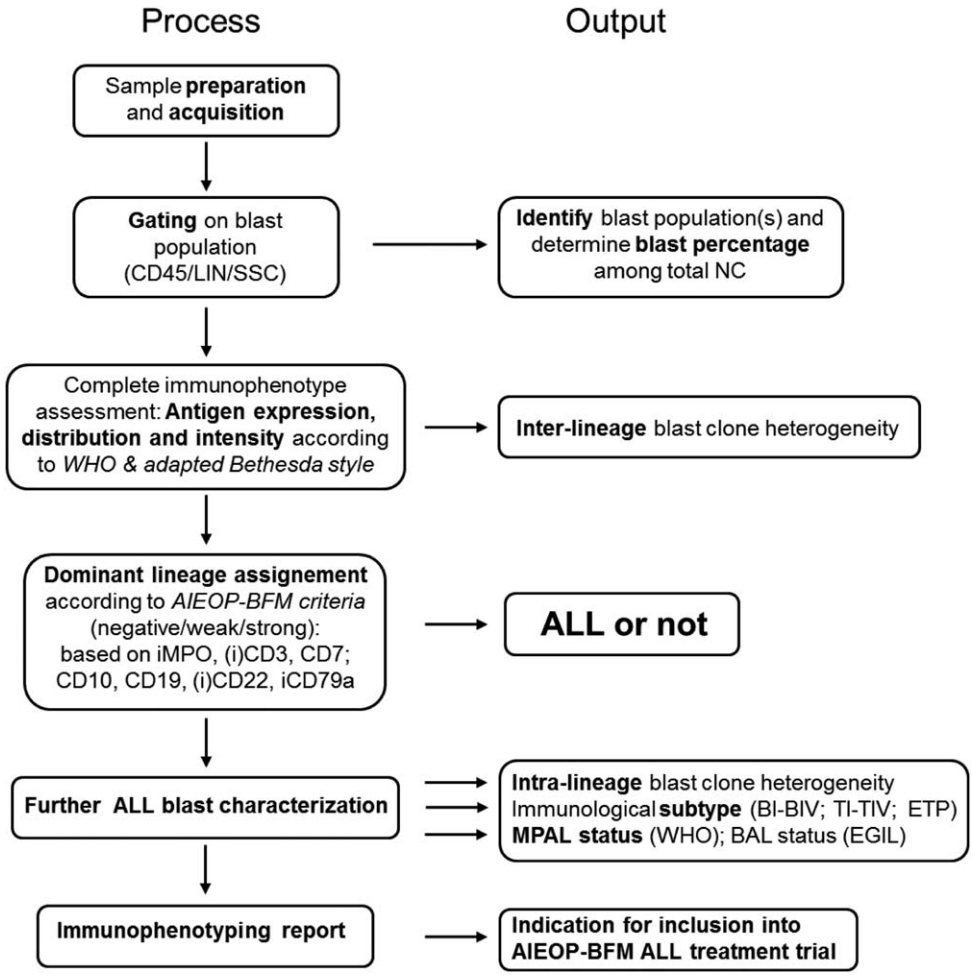
Process
Output
Sample preparation and acquisition
Gating on blast population (CD45/LIN/SSC)
Identify blast population(s) and determine blast percentage among total NC
Complete immunophenotype assessment: Antigen expression, distribution and intensity according to WHO & adapted Bethesda style
Inter-lineage blast clone heterogeneity
Dominant lineage assignement according to AIEOP-BFM criteria (negative/weak/strong): based on iMPO, (i)CD3, CD7; CD10, CD19, (i)CD22, iCD79a
ALL or not
Further ALL blast characterization
Intra-lineage blast clone heterogeneity
Immunological subtype (BI-BIV; TI-TIV; ETP)
MPAL status (WHO); BAL status (EGIL)
Immunophenotyping report
Indication for inclusion into AIEOP-BFM ALL treatment trial

FIG. 2. **Diagnostic algorithm based on the AIEOP-BFM consensus guidelines.** This algorithm is used to define the leukemic immunophenotype as prerequisite for clinical trial inclusion to AIEOP-BFM ALL trials. Note that dominant lineage assignment can lead to a diagnosis of ALL according to the AIEOP-BFM consensus despite fulfilled MPAL criteria according to WHO 2008/2016. LIN refers to usage of a lineage marker for blast cell gating as appropriate. A prefix in parenthesis indicates that the respective marker needs to be tested both on surface as well as intracellular.

Nevertheless, apart from assigning the dominant lineage of a single or simply branched leukemic clone (e.g., by partial positivity with MPO), a finding of fulfilled MPAL/BAL-criteria needs to be reported as secondary detail. Thus, dominant lineage assignment overrules French-American-British (FAB)-classification as well as MPAL/BAL designations for clinical decision making in AIEOP-BFM with the—important—exception of cases with more than one (separated or complex) blast population including a non-lymphoblastic component (see Supporting Information Fig. 6). In such case, dominant lineage assignment is not applicable as far as both separate components comprise each $\geq$10% of cells of the sample. In other words, a (probably MLL-rearranged) MPAL case with a lymphoblast subset of $\geq$10% and $\geq$10% leukemic monoblasts (of total cells) is reported as bilineal leukemia (neither as AML or ALL, therefore not to be included into AIEOP-BFM ALL trials). By contrast, if in a case of leukemia with $>$90% typical lymphoblasts an additional small subset of aberrant myeloid differentiated blasts is found, such case is designated per AIEOP-BFM rules as ALL fulfilling also MPAL-criteria and may enter the ALL protocol for reasons of clinical pragmatism. However, the admixture and type of aberrant myeloblasts—which adds the MPAL label to this case—must be described in the summary report. Notably, before accepting the MPAL label based on such suspicious cell subsets it has to be ruled out by careful phenotypic investigation that these cells just represent remaining normal hematopoiesis.

Of note, originally the EGIL did not consider B/T-BAL (9), however, newer observations notify the existence of such rare occurrences including also cases of tri-lineage differentiation (16,17,23). Hence, also such cases should be recorded as BAL using the EGIL scoring system. In B/T-BAL, the higher EGIL-rank will favor dominant lineage assignment for AIEOP-BFM ALL trial treatment arm (B or T) selection.

Cases not fitting into the ALL category will be classified as either AML, AUL (acute undifferentiated

Table 4
Contents of immunophenotyping reports in AIEOP-BFM trials

Item	Contents
Identification	Name, code number, date of birth, question/suspicion, type of sample, dates of sampling, processing/analysis, reporting, name/signature(s) of technical analyst and medical reporter
Antigen expression	"*WHO-style*" rating: *negative*, *positive weak*, *positive strong* "*Bethesda-style*" rating: as add-on, at least *partially positive*
Dominant lineage[a]	B-, T-, Myeloid-lineage independently of MPAL/BAL-status; not applicable if bilineal leukemia with non-lymphoblastic component, AUL, NK/myeloid precursor- or DC-leukemia
Subtype[b]	B-I to B-IV, T-I to T-IV; report ETP as additive result
Heterogeneities	WHO 2008 MPAL, adapted EGIL BAL criteria (also B/T and B/T/My) (Intra-lineage) blast clone heterogeneity
Quantities	Blast percentage among NC (if applicable, separate by subclones)
Conclusions	Diagnosis as relevant for treatment choice; additional information of potential clinical interest (e.g., toward underlying genetic lesions)

[a]Bilineal leukemia with a lymphoblastic and a separate non-lymphoblastic blast subset, as well as AUL, NK/myeloid precursor- and DC-leukemia are excluded from AIEOP-BFM ALL trials.
[b]Combinations of subtype labels are appropriate in case of blast subset formation at different maturation levels; B-IV with FAB-L3 morphology and *MYC*-rearrangements ("Burkitt-type") are excluded from AIEOP-BFM ALL trials, whereas B-IV cases with L1/L2-morphology and other genetic lesions are included.

leukemia), truly bilineal/"complex" immunophenotype acute leukemia, or very rare entities like NK/myeloid precursor or dendritic cell (DC)-derived leukemia (3,16). As mentioned before, these categories are not considered apt for inclusion into AIEOP-BFM ALL trials. Notably, it should be made sure to exclude also AML FAB M7 (positive for CD41, CD61, CD42b, and CD36; typically also negative for CD11a; Ref. 54).

Immunophenotypic Subtype Classification of ALL

The AIEOP-BFM consortium has a long tradition in using the EGIL-based subclassification for ALL subtyping and description of patient cohorts (8–10,55). Based on this frame, we designed an AIEOP-BFM ALL subtype classification which includes also ETP as an additional variable (Table 3). These criteria assign the leukemic subtype within a given dominant ALL lineage (Table 3) using the described AIEOP-BFM antigen expression definitions (Table 2).

The following leukemic phenotype peculiarities are recognized along the major lines of subclassification: Intracellular IgM expression is frequently found in only a subset of blasts (in 40–50% of B-III cases; see Supporting Information Fig. 4A), hence all observed differentiation stages (in populations of blasts $\geq$10%) should be reported in the subtype classification (e.g., B-II/III). In a minor subgroup of cases with iIgM expression, CD10 is negative or only weakly positive. This does not alter designation as B-III, but is recognized as frequently heralding MLL-gene rearrangements (usually NG2 positive, but not *MLL/AF4*; Ref. 13). If iIgM is only partially expressed, such cases might even be classified B-I/III. Rare cases expressing immunoglobulin light-chains (surface or cytoplasmic) occur without further features of mature Burkitt-type lymphocytic leukemia (like FAB L3-morphology, *MYC*-translocation, and a high proliferation rate/high S-phase; Refs. 56–58). Such unusual cases (or blast subpopulations) should also be given the B-IV designation as appropriate (isolated or as part of the classification, e.g., B-III/IV), and they are eligible for inclusion into conventional ALL treatment trials similar to B-I to B-III. They frequently exhibit either MLL-rearrangements or other precursor ALL-type genetic lesions (56,58,59).

In T-ALL, similar to physiologic thymocyte differentiation (60), CD3 starts to be expressed on the cell surface already in rather early maturation stages and not only in stage T-IV. Surface expression of (s)CD3 alone therefore poorly discriminates T-ALL subtypes unless strongly positive or co-expressed together with T-cell receptors. Hence, it should be emphasized that CD1a expression, irrelevant of the presence (indeed already occurring) of sCD3 defines T-III ALL in the EGIL acceptance. Moreover, T-ALL very frequently exhibits blast population heterogeneities due to immunophenotypic subclone formation, and this typically involves sCD3 (see Supporting Information Fig. 4A and B; in about 50% of T-ALLs). Likewise, CD3 surface expression (without TCR $\alpha\beta$ or $\gamma\delta$ expression) does not exclude ETP (12). This prognostically relevant new subtype is reported as additional result along with the classical T-I to T-IV subtyping. AIEOP-BFM criteria for ETP definition are stringent as per references 12,25: negative for CD1a and CD8, usually CD5 negative or weak positive, as well as strong positive for at least one of CD34, CD117, HLADR, CD13, CD33, CD11b, and CD65. In case of strong positivity of CD5, at least two of the latter must be strong positive (25,61). There is also novel evidence that the myeloid marker CD371 may be positive in ETP (compare Supporting Information Fig. 3).

Clinical Reporting

The report should allow grasping a comprehensive picture of the leukemic case as derived from immunophenotyping according to the AIEOP-BFM algorithm (see Fig. 2). This includes case identification items, the data on individual marker expression patterns, and an integrated classification of primary (dominant) lineage, as is necessary for trial inclusion and treatment selection, patterns and heterogeneities of differentiation with aspects of subset formation, as well as the quantitative aspects of the blast population(s) in the sample (Table 4). A

summary or conclusion should state the overriding diagnosis and additional information of potential clinical or diagnostic interest (for example a probable genotype association). Results should be reported in a standardized format which may make use of the FDE code (for an example see Supporting Information Fig. 6) which has been designed in particular also for database capturing of immunophenotype information (53).

DISCUSSION

In elaborating the guidelines presented herein, we considered that technical standardization alone is insufficient if not devoted to answer questions pertinent to established classification systems or clinical needs, whereas classification devoid of practical standards fosters solecism. Hence, we considered taking advantage of the possibilities of multicolor FCM by embedding it into the context of clinical trial applications. Consequently, in order to keep up with existing classification systems we needed to regulate the impact of the increased sensitivity of current multi-color FCM on the reporting. Not each minute fluorescence shift or miniscule blast subset expressing a marker should lead to results like "antigen positive" in representing the leukemic case in total. We therefore needed to choose a still artificial, but pragmatic threshold as discriminator of positive expression. It also means that in certain situations we may rate an antigen as negative in our report, oriented on the majority of blasts, but positive in a separate blast subclone of $<10\%$ which should not be overlooked and still be reported (see Supporting Information Fig. 5). More generally speaking and based on our experience with MRD assessment, we believe that also at initial immunophenotyping exists no simple cut-off regarding percentage of cells in- or excluding leukemia (compare Ref. 24). Rather, we support reporting phenotypically aberrant populations suggestive of ALL even when blast percentages are too small for a formal diagnosis of overt leukemia.

Based on the higher resolution ability of multicolor FCM, we could introduce the specification "blast clone heterogeneity" which relates to such situations where leukemic subsets with different maturation levels (or of different lineage) are seen. Commonly such phenotypic subclone formation occurs with antigens like μ-chains (iIgM), CD3, and TCR on the cell surface (Supporting Information Fig. 4), which can lead to perturbation of leukemia subclassification. As a consequence, we intensified group training and awareness on this matter to eradicate respective solecism for the future. We also specified our EGIL-based subclassification to avoid pertinent flaws in T-II vs.T-IV distinction by including TCR expression and CD3 grading into the diagnostic interpretation (Table 3). We also adapted the classification to allow for combinations of differentiation subtype-labels where appropriate (Supporting Information Fig. 4). For example, this means that we rather do not assign homogeneous leukemic subtype labels based on either highest differentiation level reached or on the larger proportion of blasts when there is a heterogeneous blast population exhibiting a differentiation drift from a subset with a lower maturation level toward one with a higher. We considered that describing rather the comprehensive subtype picture of a given ALL (like B-II/B-III) might be more useful than simplification (like quoting only B-III) in particular since subtype simplification is not necessary for any current clinical choices. Analyses of larger case cohorts are needed to clinically validate this more detailed subtyping.

The importance we put on discriminating blast cell heterogeneities in the sense of subset formation directly led to the additional nomenclature we adopted with respect to antigen distribution, as originally stated in the 2006 Bethesda recommendations (2). From this nomenclature, the annotation "partially expressed" has the most relevant impact, whereas the distinction between weak and strong expression, as suggested in the 2008 WHO guidelines, appears sufficient for most other questions asked in leukemia immunophenotyping apart from scientific analyses (3,16). It needs to be determined whether more exact, automated, and even compound expression evaluation, bar-coding, or vector-based statistical pattern transformation are of additive value (6). It will be most important for all such automation or statistical approaches not to disregard and appropriately deal with blast heterogeneities and sub-clone formation.

For a "*WHO-style*" negative/weak/strong-distinction, we realized that elaborate rules are necessary for concordant application in a consortium. Our validation data (Supporting Information Fig. 7) underscore that the approach of quantifying non-overlap of blasts with negative control renders synonymous results regarding CD19 and iCD3 expression grading compared to overlap assessment with residual antigen-positive lymphocytes as per WHO criteria. Based on this, we agreed to use the "non-overlap" approach for our grading. This is broadly applicable to many different antigens and even in samples lacking positive normal populations, either mature or immature. Immature counterpart populations have been proposed by others as gauges for rating leukemic expressions (2,44). However, often such normal cells are not found in leukemic samples at diagnosis, which hinders their use as direct in-sample comparators.

Concordant rating of antigen expressions as weak or strong is most important also for making a diagnosis of MPAL according to WHO (3,4,16). "Simple" MPAL cases (mostly BCP-ALL with a single blast clone and some MPO-positivity) are now included into the AIEOP-BFM ALL treatment strategy because it was noted that pediatric MPAL (or BAL) often does not herald a dismal outcome when it occurs together with ALL features (including findings of microscopy and genetics like *ETV6/RUNX1*- or other ALL-specific rearrangements)(17–22). Hence, we put all efforts into tracing and exploring MPAL cases fulfilling criteria of bilineal or "complex" immunophenotypes (see Supporting Information Fig. 6). Only such more extreme MPALs most probably need special therapy in children, which, however, is yet not well established. Along this line, the AIEOP-BFM

consortium puts emphasis on a diagnostic net effect, respecting all information from microscopy, genotyping, and immunophenotyping, thus reducing the relevance of MPO assessment (by FCM and/or cytochemistry) and antigen scoring (9,10) for guiding treatment choice. This is also in line with the recent 2016 update of the WHO classification where the meaning of some MPO positivity in an otherwise indisputable ALL has been diminished (4).

We emphasize that also technical issues—as shown in Supporting Information Figs. 1 and 2—are relevant causes of flaws and pitfalls in immunophenotyping (e.g., sample quality, permeabilization procedure, antibody clones, compensation, and negative control strategy). For example, we learned to do better with iCD22 assessment, which we found quite sensitive to choice of permeabilization and antibody (clone RFB4 giving best results, data not shown). Whether there is a need for more standardization (e.g., regarding antibody choice in general and combination set-up) as fostered by the EuroFlow consortium (6), will depend on outcome of our ongoing panel validation process. Instead of a full unification, it might be relevant for a consortium like ours to consider a set of common reagents against certain antigens of very high diagnostic importance or with a relevant degree of divergence when tested with different antibodies. Certain less well investigated antibodies against novel or research markers might also need a higher degree of standardization. Along this line, we are currently evaluating the impact of operator interpretation on results as well as of using a fully standardized panel as compared to local practice (manuscript in preparation). Eventually, we may also be able to establish which markers are essential for accurate classification according to our designated consensus and which could be left aside (2,62).

In conclusion, we see our guidelines presented herein as a first step of necessary inter-laboratory standardization in order to serve patients to the best we can by contemporary immunophenotyping.

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